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Fractional exhaled nitirc oxide in pulmonary hypertension

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FRACTIONAL EXHALED NITRIC OXIDE IN PULMONARY HYPERTENSION

by

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ABSTRACT

Background: Pulmonary Hypertension (PH) is a common form of high blood pressure in the lungs. It affects the pulmonary arteries, which normally allow blood to flow from the right heart to the lungs. Nitric Oxide (NO) is a potential mediator for establishing PH and decreasing its availability is implicated in the pathogenesis of PH.

Hypothesis: We tested the hypothesis that Fractional Exhaled Nitric Oxide (FeNO) is a good indicator to assess disease severity that may add to understanding the disease.

Methods: The aim of the study was to measure FeNO levels in consecutive PH patients and seek correlations with the 6 Minute walk distance (6MWD) within different World Health Organization (WHO) groups and New York Health Association Function Class (NYHA FC). Assignment to groups I or IV was done respecting the current guidelines. All values were taken at Tufts Medical Center PAH clinic visits. FeNO levels were measured utilizing the NIOX device.

Results: FeNO levels were highest in WHO Group 1 and lowest in WHO Group 5 patients. There was a strong inverse correlation between FeNO and 6MWD for each NYHA FC. (Pearson correlation of -0.986, $p = 0.014$). Within WHO Groups, we found significantly inverse correlations between FeNO and 6MWD in PH Group 4 ($p= 0.012$) and PH Group 5 ($p=0.001$). NYHA FC correlated with 6MWD across all WHO Groups ($P=0.001$).

Conclusion: We report for the first time FeNO levels in all WHO Groups of PH. FeNO levels are low in early disease. FeNO levels correlate inversely with the severity of PH in WHO Group 4 and 5 patients. The increase in FeNO in more severe patients may reflect the degree of oxidative stress and inflammation in severe PH. Further studies to determine whether FeNO may be a biomarker in early disease, especially in PH Group 4 and 5 warrants further investigation.

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LIST OF ABBREVIATIONS

ANOVA……...……....Analysis of Variance

BNP……B-Type Natriuretic Peptide

BMPR 2……..…….Bone Morphogenetic Protein Receptor Type 2

cGMP……..Cyclic Guanosine Monophosphate

COPD……Chronic Obstructive Pulmonary Disease

CTEPH…….……...Chronic Thromboembolic Pulmonary Hypertension

DLCO……..……..……..……. Carbon Monoxide Diffusing Capacity

ET-1……...…….. Endothelin-1

FeNO……..……...…....Fractional Exhaled NO

HPAH…… Heritable PAH

IPAH ……...…… Idiopathic PAH

mPAP……...……..Mean Pulmonary Arterial Pressure

NO……..…….……..…Nitric Oxide

NYHA FC……...…….…...… New York Heart Association Functional Class

PAH……..Pulmonary Arterial Hypertension

PH……. Pulmonary Hypertension

PPB……...…Parts Per Billon

RHC……...……....Right Heart Catheterization

RV……...…...Right Ventricular

SD…….……..……Standard Deviation

SSc-PAH…….……..….Scleroderma-Related PAH

WHO……..……World Health Organization

6MWD…….…..……....6 Minute Walked Distance

INTRODUCTION

Despite the increasing awareness in therapeutic advances and a greater understanding of the pathogenesis and pathophysiology in PH, the disease remains fatal if left untreated. Although there is no cure for PH, increased research in the field regarding the pathogenesis has created potential new therapeutic targets. PH is defined as a type of high blood pressure that affects the pulmonary arteries and, produces, in time right heart failure and death (27). PH leads to narrowing of the pulmonary arterioles, and capillaries (13).

In the next chapters, we will define the disease; discuss the clinical classification, clinical presentation and the involvement of the nitric oxide (NO) pathway in the pathogenesis of the disease. We will explain the fact what happens when, NO availability is decreased in PAH and NO replacing therapies are effective as therapeutic options. Lastly, we propose to investigate NO levels in the exhaled breath of PAH patients as a potential marker of the disease and its response to therapy. In addition, we are exploring NO levels in the exhaled breath on other forms of PH.

Definition and Classification of PH and PAH

The 5th World Symposium of Pulmonary Hypertension defines PH as a mean pulmonary artery pressure ≥ 25 mm Hg and a pulmonary vascular resistance of > 3 Wood Units at rest. The subtype of PAH has the same hemodynamic requirements in the setting of normal left ventricular pressures (i.e., requires a pulmonary capillary wedge pressure ≤ 15 mm Hg). Taking these parameters into consideration, the clinical classification of PH

defines 5 distinct WHO Groups (Table 1) (18). WHO Group 1, or PAH, consists of disorders that affects the small pulmonary arteries and capillaries and include idiopathic and heritable PAH, as well as PAH related to connective tissue diseases, HIV, congenital diseases, or end stage liver disease. Group 2 PH is PH due to left heart disorders (systolic or diastolic heart failure or valvular disease) and is the only group where the left sided filling pressures estimated by the pulmonary capillary wedge pressure is > 15 mm Hg. WHO Group 3 is PH due to end stage lung diseases, such as COPD or pulmonary fibrosis and is characterized by hypoxemic states. WHO Group 4 corresponds to chronic thromboembolic pulmonary hypertension (CTEPH), and WHO Group 5 is PH with miscellaneous disorders (splenectomy, sarcoidosis, hematologic malignancies, etc.).

Table 1. The Nice Classification of Pulmonary Hypertension (18).

1. Pulmonary arterial hypertension
   - 1.1 Idiopathic PAH
   - 1.2 Heritable PAH
   - 1.3 Drug- and toxin-induced PAH
   - 1.4 PAH associated with:
     - 1.4.1 Autoimmune disease
     - 1.4.2 HIV
     - 1.4.3 Portal hypertension
     - 1.4.4 Congenital heart disease
     - 1.4.5 Schistomiasis
   - 1' Pulmonary veno-occlusive disease, pulmonary capillary haemangiomatosis
   - 1" Persistent pulmonary hypertension of the newborn
2. Pulmonary hypertension due to left heart disease
   - 2.1 Left ventricular systolic dysfunction
   - 2.2 Left ventricular diastolic dysfunction
   - 2.3 Valvular disease
   - 2.4 Congenital/acquired left heart inflow/outflow obstruction and familial cardiomyopathies
3. Pulmonary hypertension due to lung diseases or hypoxia
   - 3.1 Chronic obstructive pulmonary disease
   - 3.2 Interstitial lung disease
   - 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
   - 3.4 Sleep-disordered breathing
   - 3.5 Alveolar hypoventilation disorders
   - 3.6 Chronic exposure to high altitude
   - 3.7 Developmental lung diseases
4. Chronic thromboembolic pulmonary hypertension
5. Pulmonary hypertension with unclear multifactorial mechanisms
   - 5.1 Haematological disorders: chronic haemolytic anaemia, myeloproliferative disease, splenectomy
   - 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
   - 5.3 Metabolic disorders: glycogen storage disease, Gaucher's disease, thyroid disorders
   - 5.4 Others: tumour obstruction, fibrosing mediastinitis, chronic renal failure, segmental pulmonary hypertension

Subdivision of each class under WHO classification. Table 1 classifies different diseases under each class. Adapted from Essop et al.

PAH (WHO Group 1) Subgroups

Hereditary/Familial PAH has been identified with mutations in the gene encoding the bone morphogenetic protein receptor type 2 (BMPR 2). BMPR 2 has been identified as the main cause of inherited PAH, accounting for 60% of familial cases (13, 50). Though, BMPR 2 mutation has also been identified for 10% to 25% of patients with Idiopathic PAH (IPAH) (5,13).

Scleroderma-Related PAH (SSc-PAH) is a rare autoimmune disease that has the potential to affect multiple organ systems including the gastrointestinal, cardiac, renal and pulmonary systems (40). One out of ten patients with scleroderma develop PAH, which looks similar with the IPAH and responds to the same treatments. SSc-PAH has a reported prevalence in the United States of 138 cases per million to 286 cases per million (43, 44, 48). Current guidelines recommend regular screening of SSc patients with annual echocardiography (24, 52). Other connective tissue disease patients include:

Drug and Toxin-induced PAH. Drugs, such as the appetite suppressant fenfluramine-phentermine, as well as more commonly used illegal methamphetamines have been linked with the development of PAH (42, 50). The mechanisms are unknown, but it is thought to involve the serotonin pathway via the serotonin transporter or its receptors (57).

Epidemiology of PH

The exact occurrence of all types of PH in the United States and in the world is not well known. The number of patients in the United States is certainly in the hundreds of thousands, with many more who are undiagnosed. Approximately 200,000 hospitalizations occur each year in the United States with PH as a primary or secondary diagnosis (48). About 15,000 deaths are attributed to PH per year, though this is unquestionably a low assessment (48).

Pathogenesis of PAH

One of the main pathways affected in PAH is the nitric oxide (NO) pathway, with a decrease in its bioavailability and, as a consequence, decreased vasodilation, and increased cell proliferation in the pulmonary vasculature. Proof of its importance in PH pathophysiology is the effective treatment with various NO enhancers, which either increase cyclic gualylate cyclase (cGMP) via phosphodiesterase 5 (PDE5) inhibition (sildenafil, tadalafil), or soluble gulanylate cyclase (sGC) stimulation (riociguat). Unfortunately, treatment options for PAH are not always effective and it is not clear who are the patients who will benefit from treatment with NO pathway modulators, such as sildenafil, or riociguat, in other words, those who have decreased NO availability. In addition, there is no data on other forms of PH (WHO Group 2-5), for which there is no treatment available.

Prior studies showed that the NO is decreased in patients with PAH and those with scleroderma-related pulmonary arterial hypertension (SSc-PAH), but these reports enrolled a small number of patients (30,40,43)

Our study focuses on nitric oxide (NO) levels in the exhaled breath of different forms of PH as a potential biomarker of disease severity, measured with the NIOX VERO as the fraction of exhaled nitric oxide (FeNO) (1).

Clinical Presentation and Diagnosis of PH

As new therapies are developed for PAH, the ability to screen, accurately assess disease severity, and provide a prompt diagnosis have been essential to treating this disease. PH/PAH should be suspected in patients that present with symptoms of unexplained dyspnea on exertion that progresses over time, syncope, and/or signs of right ventricular dysfunction (4, 31). Diagnosis can be delayed due to subtle findings and non-specific symptoms experienced during physical examination. Symptoms that are more indicative of advanced PH/PAH include exceptional chest pain and lower extremity edema (10). Women are more likely to be affected by PAH than men and patients of all ages can develop this disease (31). The mean age of diagnosis is between the ages of 36 to 50; with more recent studies including associated PAH (specifically scleroderma) which has reported an older mean age of diagnosis (5, 32, 37).

The degree of dyspnea on exertion is quantified by the New York Heart Association Functional Class (NYHA FC), Table 2 (34,35). The higher the NYHA FC, the worse the prognosis (51). Therefore, correlations between NYHA FC and novel potential biomarkers such as FeNO are essential to being explored.

Table 2. New York Heart Association of Functional Classification (34).

Functional Class	Patient Symptoms
I	No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea (shortness of breath).
II	Slight limitation of physical activity. Comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea (shortness of breath).
III	Marked limitation of physical activity. Comfortable at rest. Less than ordinary activity causes fatigue, palpitation, or dyspnea.
IV	Unable to carry on any physical activity without discomfort. Symptoms of heart failure at rest. If any physical activity is undertaken, discomfort increases.

Description of what a patient's functional ability would be under each functional class.

Prognosis

There has been an increased interest for clinically relevant prognostic predictors, which has been essential in the diagnosis, prognosis, and type of therapy patients with pulmonary hypertension should receive. National surveillance data indicates that there

has been an increase in mortality in patients with PH. The data states that there has been an increase in mortality from 5.2 to 5.4 per 100,000 over a 22-year period (1980-2002). The data also shows that the greatest increase has been found in both African-Americans and women. (38)

Several parameters are associated with a poor prognosis. The NIH was the first to show the impact of baseline hemodynamics on risks of death. They showed that factors governing right heart function could determine prognosis. They created a formula, which included the measurements of right atrial pressure, cardiac, index and mean pulmonary artery pressure (11). However, it has become clear that no single parameter can fulfill the role of a reliable prognostic indicator. Recent studies involving two large registries have shed light on the prognosis of patients with PAH in the current therapeutic area. The French registry followed patients with IPAH patients and demonstrated that there was an improvement in survival of this cohort of PAH patients compared to the predicted survival on the basis of the NIH registry. However, it was suboptimal, with 1, 2, and 3-year survival of 85.7%, 69.5% and 54.9% respectively for incident cases (36). The French registry identified that important predictors of survival included sex, functional class, and exercise tolerance—which is measured by 6MWD and hemodynamics, specifically right arterial pressure and cardiac output. Similarly, the United States based registry classified the same predictors as the French registry but added etiology of PAH. Rather than focusing on right arterial pressure for hemodynamics, the United States registry focused on hemodynamics that reflected right ventricular function (8). More

recent studies conducted by Nickel et al. reported a systemic evaluation of prognostic markers at baseline and follow up in a series of patient with IPAH (51).

Diagnostic Evaluation. As stated by Hoeper et al, a comprehensive evaluation is needed in order to establish the diagnosis and etiology of PAH. The evaluation includes pulmonary function testing, connective tissue disorder serology, echocardiography, and a ventilation/perfusion scan. The definitive diagnosis of PAH also requires a right cardiac catheterization to accurately measure right-sided pressures and cardiac impairment (4, 31). Echocardiography is widely used for the initial evaluation of PH, although it has been shown to be inaccurate for the definitive diagnosis (51, 62). An echocardiograph allows non-invasive estimation of the pulmonary artery systolic pressure of a patient by utilizing Doppler techniques, which has been shown to have an excellent overall correlation (28, 55). However, there has been a large standard of error (5 to 8 mm Hg) and the results should only be viewed as an estimate rather than an actual value of pulmonary arterial pressure (9, 17). An echocardiograph can show findings such as an enlargement of the right heart chamber, as well as the left ventricular surface area, tricuspid insufficiency, and the paradoxical motion of the interventricular septum (13). If there is no connective tissue disease, and pericardial effusion is visualized, this may indicate a correlation with elevation in right arterial pressure (13). Although an echocardiograph allows for a good understanding of whether or not a patient may have PH/PAH, right heart catheterization (RHC) is still the gold standard for diagnosis. Right heart catheterization provides measurements of pulmonary arterial pressure, cardiac output, right atrial pressure, and pulmonary wedge pressure (28, 54, 55). It also examines

left-sided heart disease and the potential correctable intracardiac left to right shunting (31). RHC is an invasive biopsy procedure that can be physically demanding and is associated with serious and sometimes fatal complications (33). Due to RHC being an invasive and not a routinely conducted procedure, it is recommended that the procedure be done in a specialized center (11, 22). A diagnosis of PAH requires the exclusion of other causes of PH. Hoeper et al proposed a diagnostic algorithm that can be seen in Figure 1 (31).

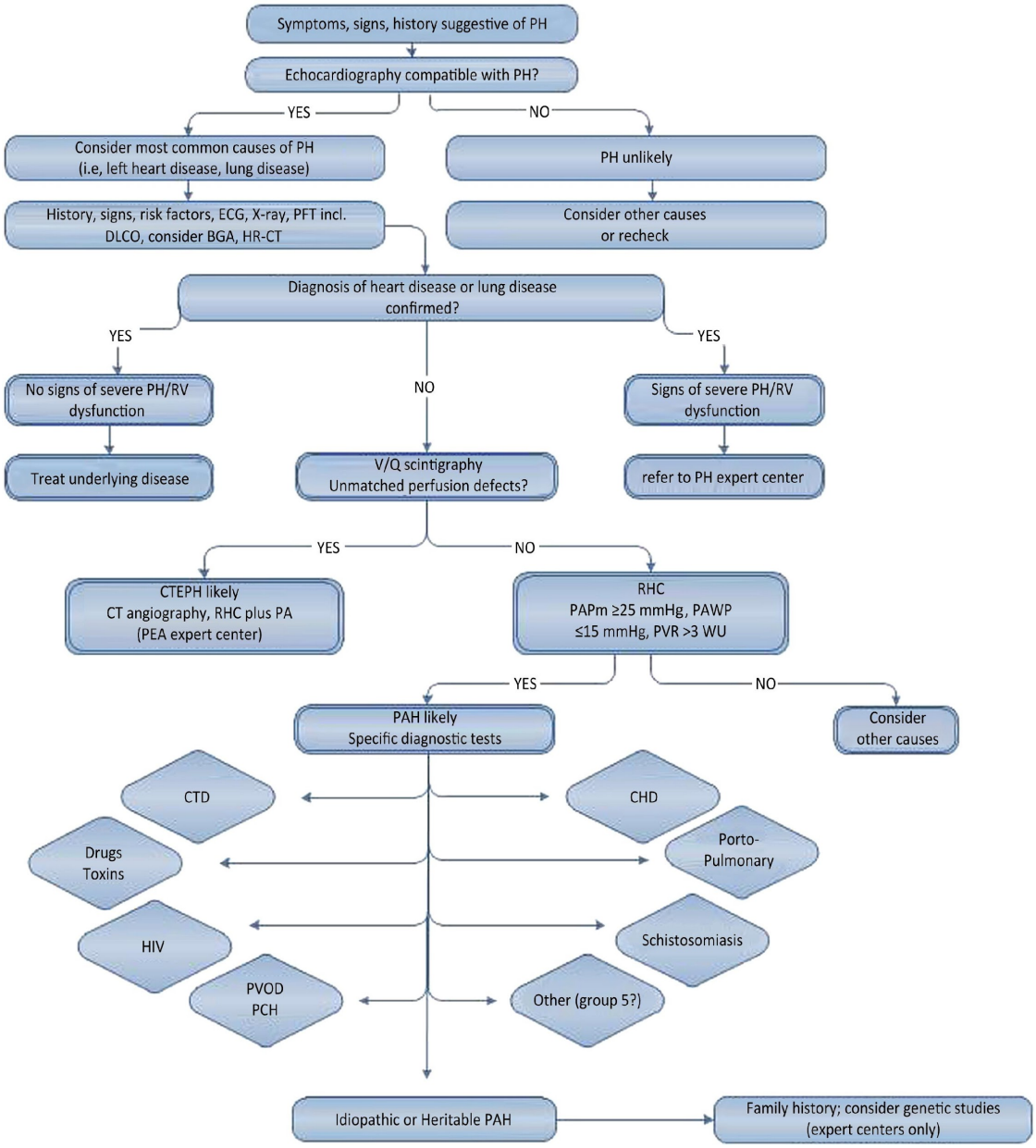

Figure 1. Diagnostic Approach to Pulmonary Hypertension (31). This algorithm demonstrates, a diagnostic approach to how to properly diagnose each PH patient due to the complexities of the disease. Adapted form Hoeper et al.

Diagnostic Tools (also used as markers of prognosis in PAH).

6-Minute Walk Distance. 6MWD is a simple, inexpensive and noninvasive test, which is easily reproducible. The 6MWD exam is well tolerated in PAH patients and studies have shown significant correlation between baseline 6MWD and hemodynamic parameters, as well as survival (49). 6MWD has become an endpoint for many of the pivotal clinical trials, which led to the approval of PAH therapies, including sildenafil, tadalafil and riogiguat. 6MWD has served well as an endpoint but has also been a predictor of functional state and survival (46, 49, 61).

6MWD have been widely used by clinicians to assess prognosis at baseline and treatment results at follow-ups (45). Though the 6MWD has become popular, it has some limitations. These include learning effects, day-to-day variation, and the impact of demographics, characteristics, and comorbidities (45). Another limitation is the inability to detect meaningful changes in 6MWD with the use of combination treatment therapies (16, 42, 58). In a study conducted by Savarese et al., the results of tests on 3,112 patients from 22 clinical trials demonstrates that the treatment for PAH shows a significant reduction in all causes of death (60). These favorable results due to clinical intervention were not predicted by changes in 6MWD.

BNP. Brain natriuretic peptide (BNP) is released by the distended atria in the setting of heart failure. BNP and its precursor, N-terminal-proBNP (NT-proBNP), correlates with the degree of heart failure and has emerged as a secondary endpoint in PAH trials. Current guidelines set by the WHO suggest a “normal” BNP level as a potential treatment goal. BNP levels have paralleled hemodynamic and functional

responses to PAH therapies in most clinical trials (45). A study conducted by Nickel, N-terminal pro–B-type natriuretic peptide levels carries prognostic information. The study showed that baseline and follow-up NT-proBNP levels <1,800 pg/ml indicate a better chance of survival in a cohort of 84 PAH patients in the current treatment era (51).

Pulmonary Function Testing. The role of carbon monoxide diffusing capacity (DLCO) as a non-invasive measure of PAH has been a potential indicator of prognosis of patients with PH/PAH. It was found that a DLCO less than 39% in those diagnosed with PAH was associated with a four-fold increase in the risk of death (15). In a study conducted by Chung et al. stated that a NYHA FC IV status and a severely decreased DLCO at the time of PAH diagnosis portend a poor prognosis particularly with older patients. Another study conducted by Fisher et al. utilized a bivariate analysis to identify that a decreasing DLCO is associated with a higher mortality. Fisher continued to state that a severely altered DLCO might be an indicator of an undiagnosed interstitial lung disease and/or significant pulmonary vascular disease (20). The above studies indicate the importance of monitoring DLCO for screening, diagnostic and prognostic purposes.

Natural History.

The NIH was the first to show the impact of baseline hemodynamics on risks of death. They showed that factors governing right heart function could determine prognosis. They created a formula, which included the measurements of right atrial pressure, cardiac index, and mean pulmonary artery pressure. However, it has become clear that no single parameter can fulfill the role of a reliable prognostic indicator (30). Recent studies involving two large registries have shed light on the prognosis of patients

with PAH in the current therapeutic area. The French registry followed patients with IPAH patients and demonstrated that there was an improvement in survival of this cohort of PAH patients compared to the predicted survival on the basis of the NIH registry (7).

In general, in the absence of therapy, prognoses for patients in PAH Group 1 have a poor chance of survival. The United States registry reported from the time of RHC patients with IPAH, had a 1, 3, 5 and 7-year survival rate of 85%, 68%, 57% and 49%, respectively (7). A study conducted by Chung et al. compared IPAH to patients with a connective tissue disorder like SSc-PAH patients. SSc-PAH patients showed poorer outcomes and had an estimated survival rate of 1 and 3 years of 72-86% and 39-67%, respectively (15). Chung also stated in her study that a severely decreased DLCO and a NYHA FC of IV status at the time of PAH diagnosis resulted in a poorer prognosis especially in older male patients.

Taken together, a multitude of parameters most of them requiring invasive monitoring, are necessary to determine prognosis. Therefore, additional biomarkers to help determine the severity of the disease as well as response to therapy are needed.

FeNo. A decrease in NO has been considered an important pathogenic mechanism in PAH. NO is produced by endothelial cells and has been shown to be crucial in maintaining a low vascular tone in the pulmonary arteries (21,41,53). NO production is oxygen dependent and lack of NO synthesis under hypoxic conditions, such as chronic obstructive pulmonary disease (COPD), is associated with pulmonary vasoconstriction

(12,14). This can eventually lead to PH (53, 59). Vasoconstriction has, in turn, been shown to cause pulmonary arterial wall remodeling (6, 19, 63).

Prior studies showed that exhaled NO levels are decreased in patients with PAH and those with scleroderma-related pulmonary arterial hypertension (SSc-PAH). (2,39,56)

Treatment

Pharmacologic agents used in treatments of PAH include calcium-channel blockers, prostanoids, endothelin antagonists, and phosphodiesterase type 5 inhibitors or soluble gualylate cyclase stimulators—which act on the NO pathway (41). The goal of therapy aims to decrease a patient's risk of death, to achieve a lower NYHA FC, improve their exercise capacity performance on 6MWD, and to improve hemodynamics (45). A summary of the variables used in clinical practice to assess response to therapy can be seen in Table 3. All these parameters have limitations as functional biomarkers.

PAH therapies have shown to improve both patients NYHA FC and 6MWD. A list of these medications as well as common dosage regimens can be seen in Table 4

Table 3. Variable Used in Clinical to Determine Response to Therapy and Prognosis in Patients with PAH (45)

NYHA Functional Class I or II
Echocardiography/CMR Normal/near-normal RV size and function
Hemodynamics Normalization of RV function (RAP <8 mm Hg and CI >2.5 to 3.0 l/min/m^2)
6-min walk distance >380 to 440 m; may not be aggressive enough in young individuals
Cardiopulmonary exercise testing Peak VO_2 >15 ml/min/kg and $EqCO_2$ <45 l/min/l/min
B-type natriuretic peptide level Normal

CI = cardiac index; CMR = cardiac magnetic resonance; $EqCO_2$ = ventilatory equivalent for carbon dioxide; PAH = pulmonary arterial hypertension; RAP = right atrial pressure; RV = right ventricular; VO_2 = peak oxygen consumption.

Different types of assessments in order to analyze ones disease progression and response to therapy. Adapted from McLaughlin et al.

Table 3. Medical Therapies for PAH (13)

PH Medication	Dose
Bosentan (Tracleer)	62.5 mg by mouth twice daily Increase to 125 mg after 4 weeks use twice daily
Ambrisentan (Letairis)	5 mg by mouth daily; consider increasing to 10 mg daily if 5 mg is tolerated
Sitaxsentan (Thelin)*	100 mg by mouth daily
Sildenafil (Revatio)	20 mg by mouth 3 times daily
Beraprost*	20 µg by mouth 4 times a day; increase in increments of 20 µg 4 times a day if tolerated
Iloprost (Ventavis)	2.5 µg inhaled 6 to 9 times daily; if tolerated increase to 5 µg 6 to 9 times daily
Epoprostenol (Flolan)	2 ng/kg/min and increase every 15 min until dose-limiting side effects occur †
Treprostinil (Remodulin)	1.25 ng/kg/min and increase by no more than 1.25 ng/kg/min weekly for 4 weeks and after that by no more than 2.5 ng/kg/min weekly

*Not approved in the U.S. † Intravenous epoprostenol and treprostinil dosing vary; the epoprostenol package insert goes on to say that *more commonly* a slower titration schedule is used. Typically dose increases of 0.5 ng/kg/min to 1 ng/kg/min are performed initially daily and then approximately weekly until the target dose is achieved, limited by side effects.

All the various PH medications that are used to potential treat PH for each WHO and NYHA FC classification. Adapted from Chin et al.

All these agents have pulmonary vasodilation effects and all, except calcium-channel blockers, are thought to have anti-proliferative properties (13). Epoprostenol remains the treatment of choice for most of NYHA FC IV patients. Oral therapy has been the initial treatment of choice for patients within the NYHA FC II and III (3). The endothelin antagonist, Bosentan, is a nonspecific endothelin antagonist that blocks both endothelin A and B. Bosentan is used to block pulmonary vasoconstriction induced by ET-1 and partially blocked acute hypoxic vasoconstriction (29). Bosentan therapy

reported a subjective improvement in symptoms, 6MWD increased from 296± 34 meters to 341±41 meters. Bosentan also showed an improvement in FeNo at lower expiratory flow rates (26). Phosphodiesterase type 5 inhibitors, like sildenafil, increase the effects of locally produced NO by inhibiting the breakdown of NO's secondary messenger cyclic guanosine monophosphate (cGMP), resulting in vasodilation and inhibition of smooth muscle cell growth (13). Treatment with sildenafil has shown improvement in symptoms, 6MWD, and hemodynamics. One study even showed 95% survival rate after one year (23). Five-year survival among epoprostenol treated patients is now between 47% to 55%, with a greater than 70% survival rate if the patient can improve into a NYHA FC I and II as shown in Figure 2 (47). Studies have shown survival advantages with epoprostenol with observed survival rates in 1, 2, 3, 4 and 5 years of 88%, 76%, 63%, 56%, and 47%, respectively. Lastly Gaille et al. suggested that active treatments were associated with a reduction in mortality of 43% (25).

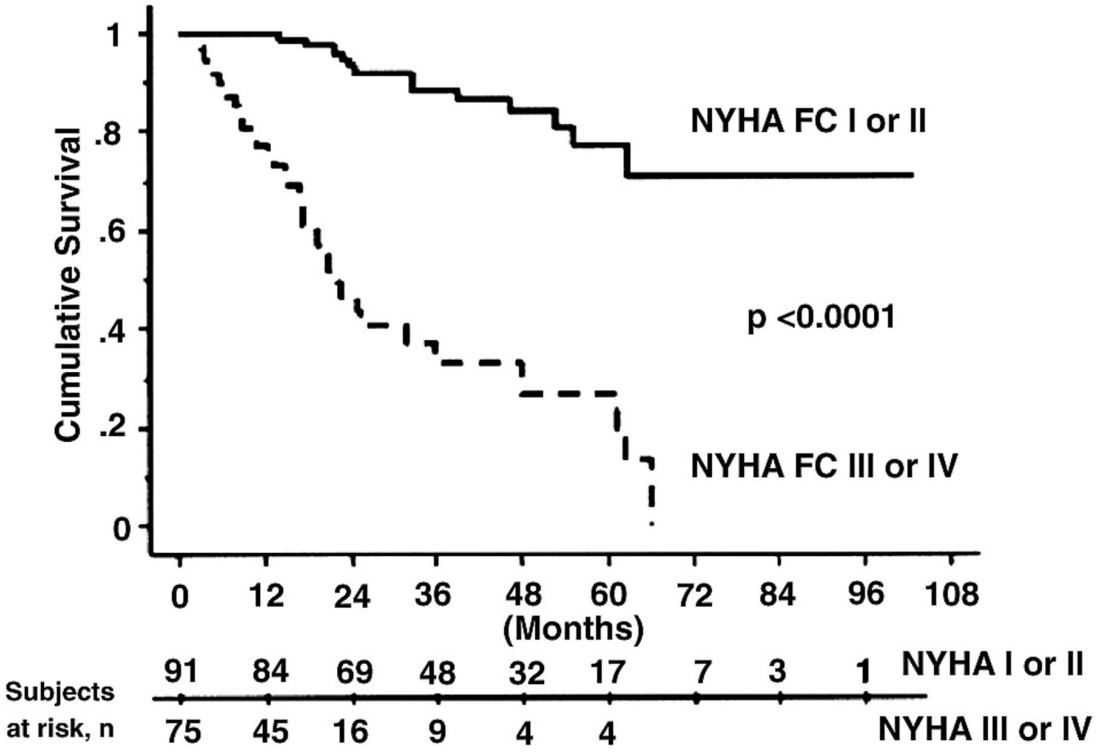

Figure 2. Survival in Patients with Idiopathic PAH After Treatment with Epoprostenol (13). Adapted from Chin et al. Among patients who were NYHA FC III or IV prior to treatment with epoprostenol, survival among patients improving to FC I or II (solid line) was substantially better than patients who were FC III or IV (dashed line) after treatment; p < 0.001. NYHA = New York Heart Association

HYPOTHESIS

It is hypothesized that in a population of PAH patients, exhaled NO levels (FeNO) are low and correlate with disease severity, as determined by the impaired NYHA FC and 6MWD. In addition, the following research is aimed to evaluate FeNO levels in all WHO Groups of PH and compare them with PAH Group 1.

The long-term goal is to better define FeNO in PAH and determine its role as a biomarker and to assess FeNO in all forms of pulmonary vascular disease and its impact to disease activity.

METHODS

Patient Population. This study tested consecutive PH patients who were evaluated at Tufts Medical Center PH clinic from September 2016 to February 2018. All patients underwent full clinical evaluation, including a right heart catheterization for complete assessment and were classified, according to the WHO groups, by expert PH physicians at Tufts Medical Center. The protocol was approved by the Investigational Review Committee at Tufts Medical Center. Patients gave written informed consent to be included in the PH database. At each clinic visit patients underwent a 6MWD and an FeNO measurement, as well as an assessment of the NYHA FC by an experienced physician.

FeNO Measurements. NIOX VERO is a small hand-held and portable device, which can be used on both children and adults. It requires a 10 second exhalation of breath by the patient, at an exhalation pressure of 10-20 cm H2O to maintain a flow rate of 50±5 mL/s (1). A calibrated electrochemical sensor provides a definitive result in parts per billion, which analyzes the last 3 seconds of the 10-second exhalation. Clinical cut-off values can be applied to the FeNO values to categories reading as low, intermediate, or high according to the reference ranges for age (1). Exhaled nitric oxide values were taken prior to 6MWD, to exclude the effect of exercise on NO production. Normal levels are considered 27- 57 PPB (1).

Six Minute Walk Test. The 6MWD was performed according to the American Thoracic Society criteria and was measured in meters. The 6MWD was performed indoor, along a long, flat, straight, 30 meter enclosed corridor with a hard surface. The

patient is at rest for a least 10 min prior to testing. Before starting each 6MWD the patient's blood pressure, heart rate, level of fatigue, dyspnea based of the Borg scale, and oxygen saturation were measured (53). The patient was then instructed to walk as long as they think they possibly could. They were asked to indicate if they were under any discomfort or if they felt as if they could no longer do the testing. The patient's performance was measured by the number of laps and total distance walked along with the amount of times the patient took a break during testing. Immediately after the six minute test is completed, the patient's blood pressure, heart rate, level of fatigue, dyspnea level, and oxygen saturation were recorded again. The NYHA FC was recorded during clinical assessment, according to Table 2. Demographic characteristics were presented as descriptive values. Correlations between FeNO and 6MWD and NYHA FC were performed.

Statistical Analysis. Data is presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to perform comparisons between different groups. Correlations between FeNO and 6MWD/NYHA FC were evaluated by Pearson correlation. Means ± SD were compared via linear regression analysis. Minitab version 18.1 (Pennsylvania State University, PA, USA) was used for statistical analysis as well as all linear graphs, bar graphs. Significance was established at $p < .05$.

RESULTS

Patient Demographics. One hundred and six consecutive tests were run on seventy-five patients. Table 5 shows baseline demographics, clinical data, WHO Group, NYHA FC, FeNO, and 6MWD obtained. Tables 6 and 7 compare the same information in prevalent and incident patients, respectively. It is found that, in general, patients in NYHA FC II or III were middle aged, mostly females and were symptomatic. The group consisted mostly from prevalent patients. 6MWDs were low in all groups, especially in WHO Group 3, which showed significantly lower 6MWD at an average of 313.61 ± 144.61 meters compared to the other four WHO groups that had > 400 meters in each class.

Table 4. Patient Demographic Characteristics and Results of Functional Testing

WHO Group	1	2	3	4	5
Patient (n)	52	20	16	9	9
Age mean, (SD)	58.81 (12.5)	61.2 (13.8)	66.93 (10.72)	56.4 (13.31)	55.62 (10.13)
Female: Male (n)	38:14	9:11	9:7	1:8	6:3
NYHA FC (n) I II III IV	 13 26 12 1	 2 11 7 0	 0 5 11 0	 2 7 0 0	 1 2 6 0
6MWD (Meters) Mean (SD)	447.03 m, (128.42)	438.96 m, (142.07)	307.89 m, (147.36)	486.57 m, (66.2)	449.91 m, (142.91)
FeNO (ppb) Mean (SD)	20.02 (18.43)	20.15 (10.5)	20.38 (16.38)	16.11 (9.46)	15 (7.50)

WHO= World Health Organization; NYHA FC= New York Health Association Functional Classification; 6MWD= 6 Minute Walk Distance; FeNO= Fractional Exhaled Nitric Oxide. The whole cohort had a total of 75 patients with 106 tests run overall

Table 5. Patients Demographic Characteristics and Results of Functional Testing on PAH Medication (prevalent patients).

WHO Group	1	2	3	4	5
Patient (n)	44	10	9	6	6
Age mean (SD)	58.6 (11.6)	67.4 (8.4)	66.3 (13.5)	56.8 (12.8)	52.6 (11.5)
Female: Male (n)	30:14	4:6	5:4	1:5	5:1
NYHA FC (n) I II III IV	 10 24 9 1	 0 5 5 0	 0 3 6 0	 1 5 0 0	 1 1 4 0
6MWD (Meters) Mean (SD)	435.8 (135.7)	396.2 (157.2)	259.1 (98.2)	468.3 (53.5)	455.7 (76.4)
FeNO (ppb) Mean (SD)	20.27 (19.68)	22.90 (13.21)	25.44 (19.07)	18.5 (10.21)	15.0 (4.43)

(Abbreviations refer to Table 5.) A total of 75 tests were run on patients that were on PH medication. Patients on PH medication had higher FeNO and the lower 6 MWD compared to the patients that were not on PH medication.

Table 6. Patient Demographics Characteristics and Results of Functional Class Not on PAH Medication (treatment-naive, incident patients).

WHO Group	1	2	3	4	5
Patient (n)	9	10	6	3	3
Age mean (SD)	61.1 (16.5)	55 (15.7)	67.5 (7.3)	58.6 (4.93)	55.7 (17.21)
Female: Male (n)	8:1	5:5	4:2	1:2	0:3
NYHA FC (n) I II III IV	 1 6 2 0	 2 7 1 0	 0 2 4 0	 0 2 1 0	 1 2 0 0
6MWD (Meters) Mean (SD)	480.6 (117.9)	481.7 (117.7)	342.8 (183.0)	523.0 (86.0)	532.8 (54.0)
FeNO (ppb) Mean (SD	17.44 (9.8)	17.40 (6.54)	13.17 (9.77)	11.33 (6.81)	12.00 (4.58)

(Abbreviations refer to Table 5.) A total of 31-test were on patients that were not on PH medication. Patients not on PH medication showed lower FeNO and higher 6 MWD compared to patients on PH medication.

FeNO. FeNO levels in different WHO Groups and NYHA FC are presented in Figures 3 and 4, respectively. FeNO levels tended to be lower than normal in all WHO Groups, especially in WHO Groups 4 and 5 and in treatment-naive patients.

FeNO levels were similar across NYHA FCs I-IV (p=0.06), although a clear conclusion cannot be obtained, as there was only one patient in NYHA FC IV.

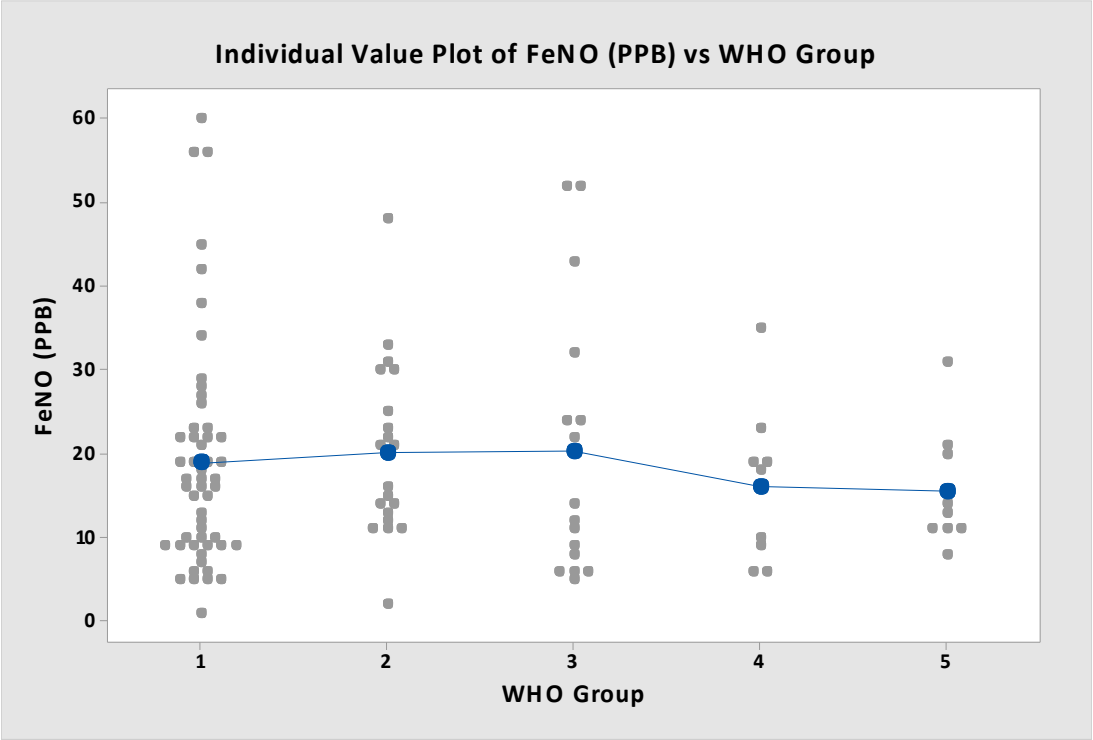

Figure 3. The dots represent FeNO (PPB) levels for the 5 WHO groups for the active cohort. There was no statistical significant difference between the groups. Blue dots represents the mean values for each group. WHO Group 3 had the highest FeNo compared to Class 5 who had the smallest FeNo.

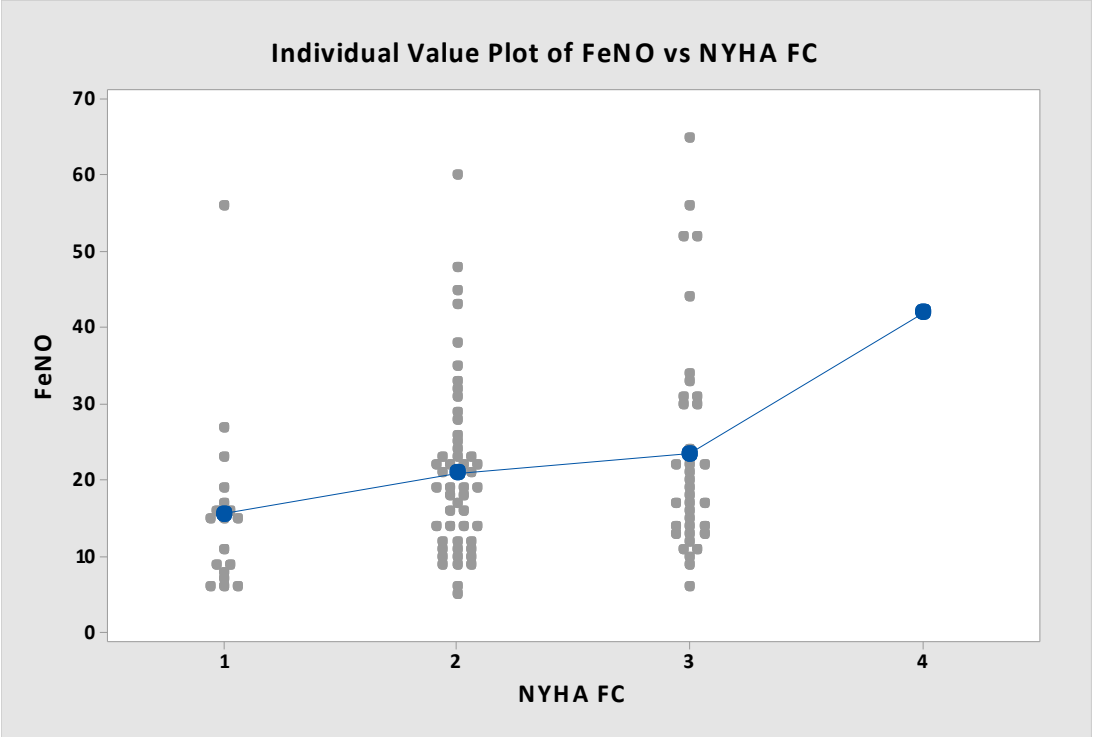

Figure 4. FeNo (PPB) for NYHA FC classification for the active cohort. There was no statistical significant difference between the four classifications. Box plot descriptions can be seen in Figure 3. NYHA FC class III had the highest FeNO compared to class I who had the smallest FeNO. It can be seen that a trend in NYHA FC trends upwards as one progresses

6MWD per WHO Group. In this cohort, patients in PAH Group 1 performed best on average, while patients in WHO Group 5 had the most limitations (Figure 5). This is the first time a significant difference in 6MWD is found between WHO Groups.

6MWD per NYHA FC. As previously reported, the more symptomatic the patients, the less they were able to perform their hallway walk test (Figure 6). As stated earlier, NYHA FC IV had the lowest 6MWD of 141 meters but only has a sample size of one.

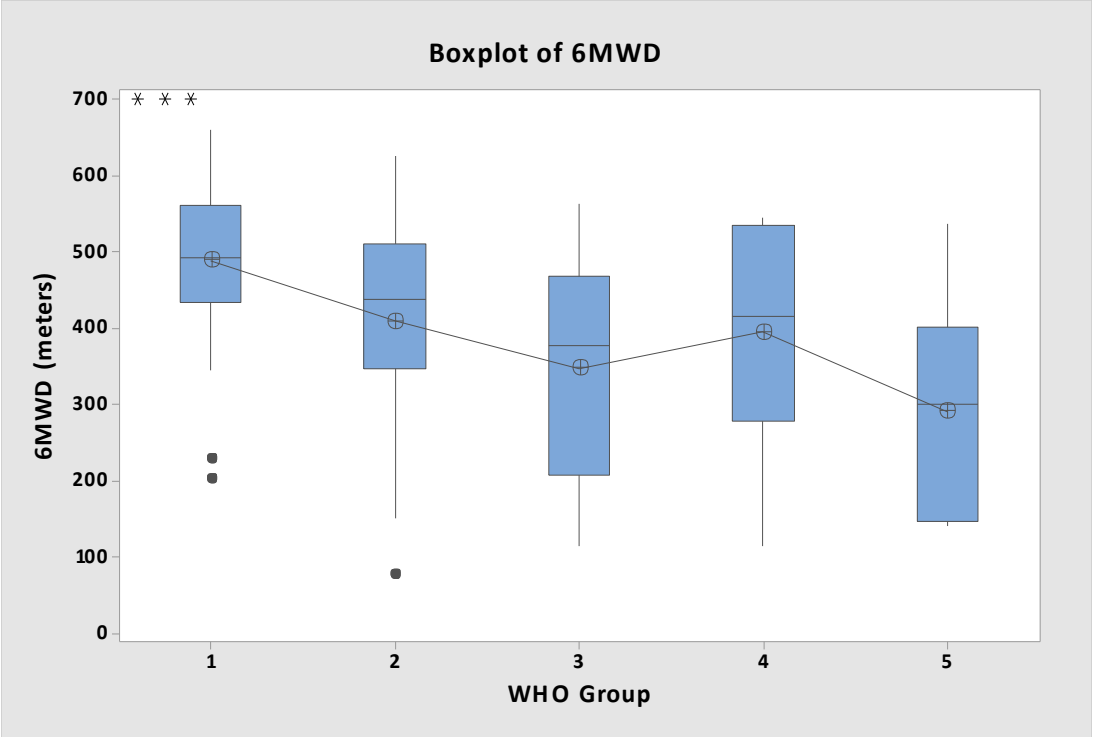

Figure 5. 6MWD (expressed as meters) for the five WHO Groups. The horizontal line represents median value. The cross hair represents the mean value and the box plot indicates 75% of the data for each class. WHO Group 3 had the lowest 6MWD compared to other classes who all walked more than 380 meters. There were significant differences between the WHO groups (*p <0.001)**

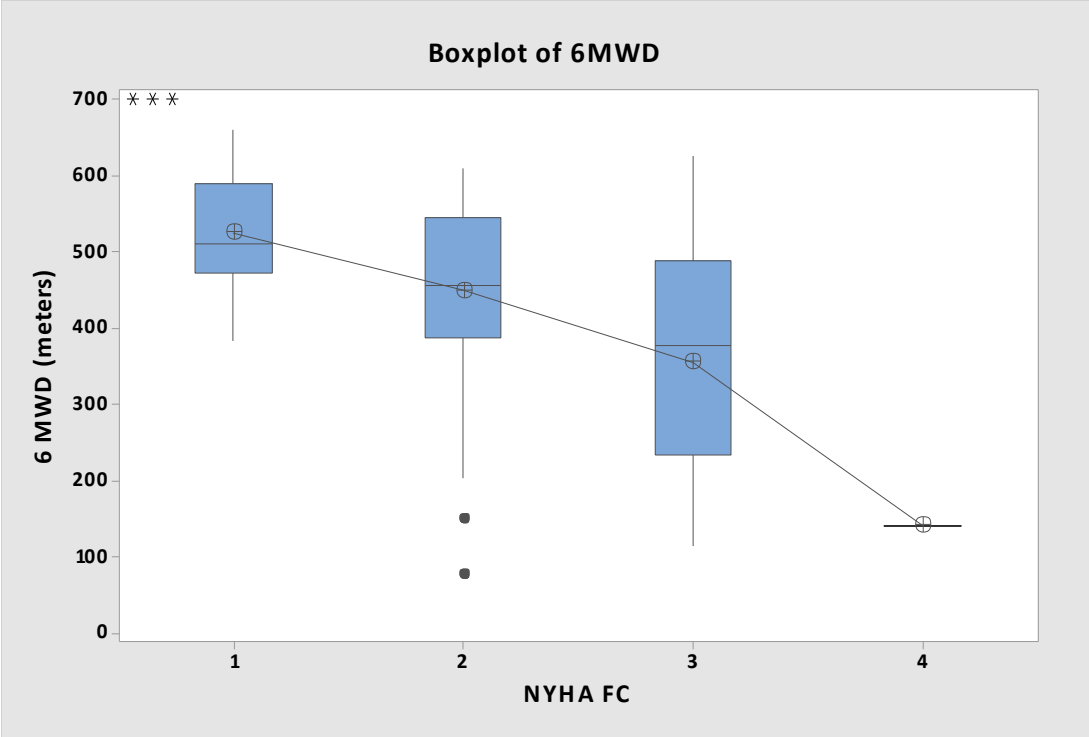

Figure 6. 6MWD (expressed as meters) for NYHA FC classification. See Figure 5 for stats (*p <0.001) NYHA FC had the lowest 6MWD, 6MWD decreased in NYHA FC patients with a statistically significant different. Class IV had a sample size of one.**

FeNO vs 6MWT. Linear regression analyses were run for both WHO and NYHA FC, comparing the means of FeNO to 6MWD within the WHO Group (Figure 7), or within the NYHA FC (Figure 8). There was no significant correlation within the WHO Group. Conversely, the higher the 6MWD the lower the FeNO. Therefore, there was as strong inverse correlation between FeNO and 6MWD for each NYHA FC.

FeNO in different PH etiologies (WHO Groups). Within the WHO Groups, there are significantly inverse correlations between FeNO and 6MWD in PH Group 4 (CTEPH, Figure 9) and PH Group 5 (Figure 10). There were no correlations within other PAH Groups.

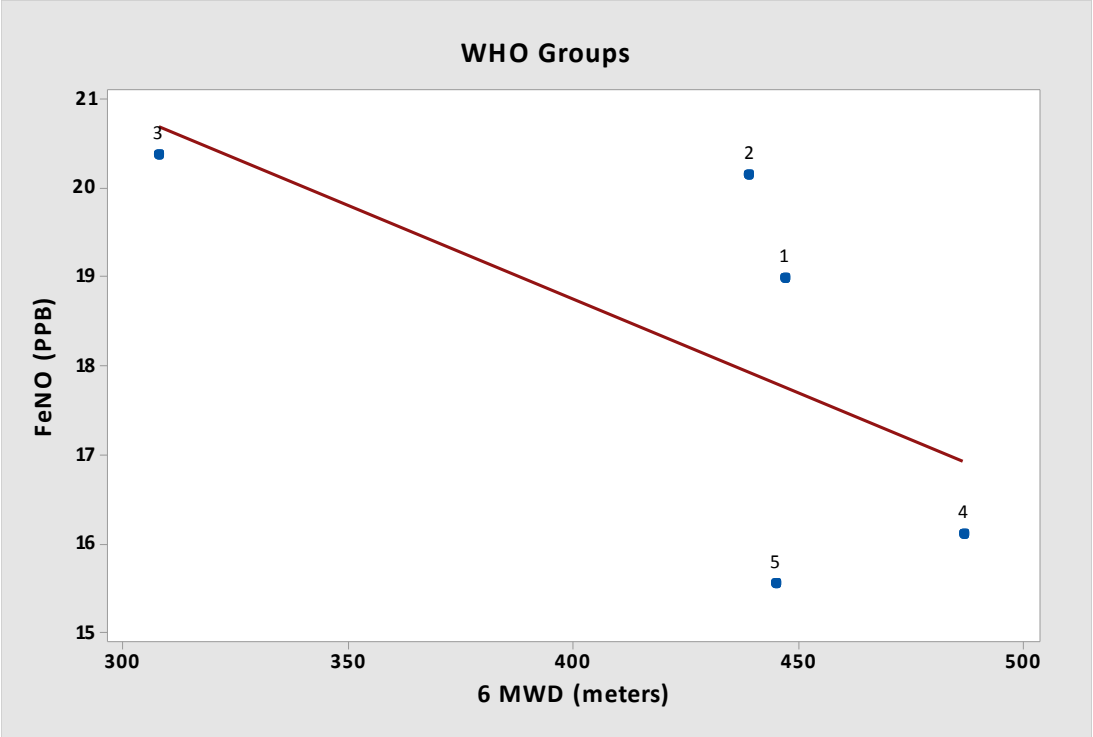

Figure 7. FeNo (ppb) vs 6MWD (meters) displays means for WHO Group 1 through 5. Linear regression shows a strong negative Pearson correlation of -0.635 with a p value=0.250.(Non Significant)

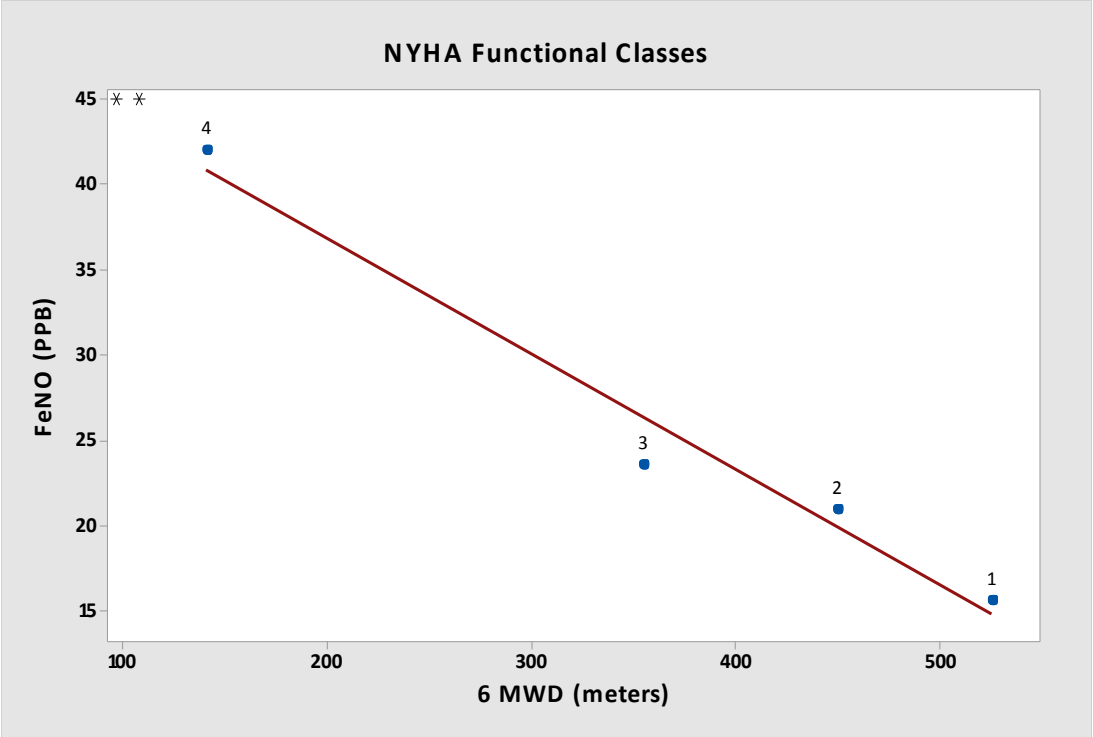

Figure 8. FeNo (PPB) vs 6MWD (meters) displays means for NYHA FC I-IV. The higher the NYHA FC, the higher the FeNO, as the disease is more severe. Linear regression shows a very strong inverse Pearson correlation of -0.986, and a significant ** p value of 0.014.

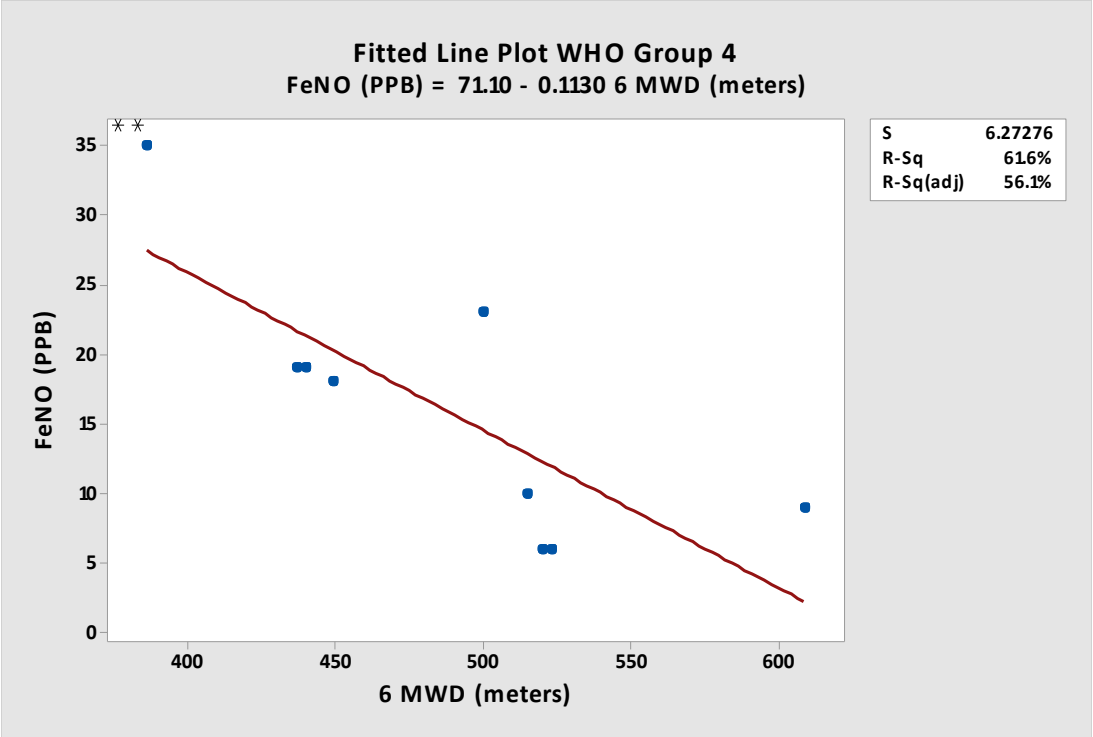

Figure 9. FeNO (ppb) vs 6MWD (meters) displays for WHO Group 4. As one can see in each patients for WHO Group 4 FeNo: Linear regression shows a strong inverse correlation between FeNO and 6MWD for WHO Group 4. (N =9; r = 0.616; **p value=0.012.

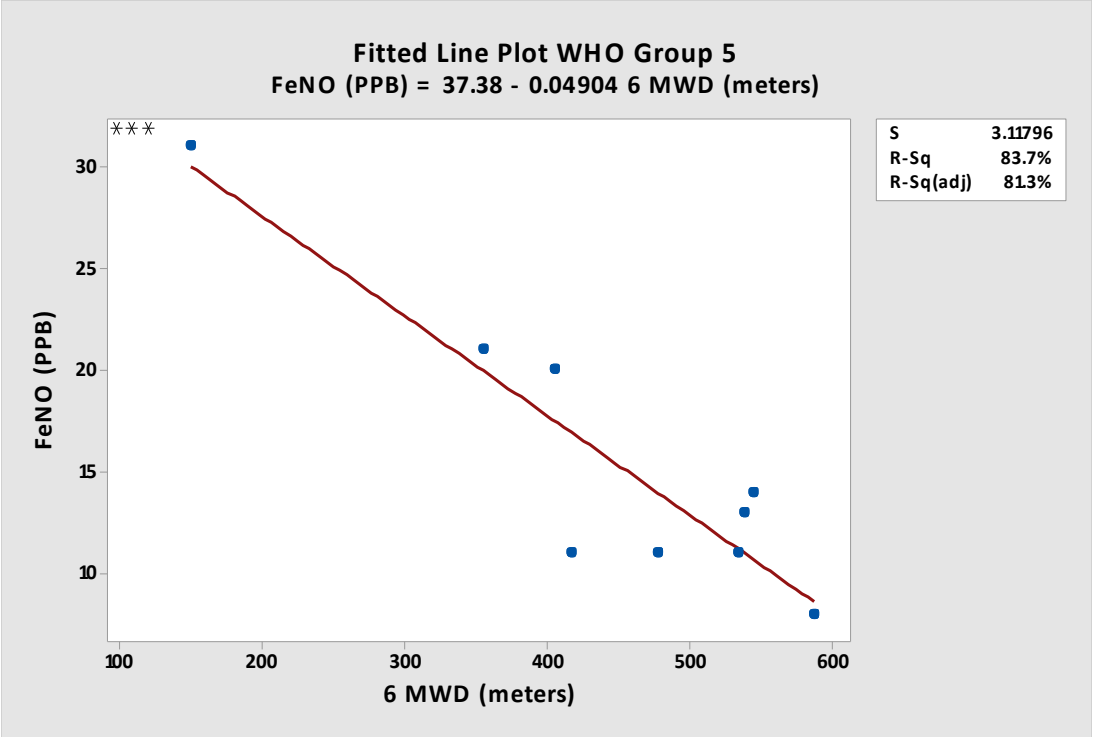

Figure 10. FeNo (ppb) vs 6MWD (meters) displays for WHO Group 5. As one can see in each patient for WHO Group 4 FeNO: Linear regression shows a strong inverse correlation between FeNO and 6MWD for Class 4. (N =9; r = 0.837; *** p value=0.001.

DISCUSSION

The status of exhaled NO in PAH/PH is unclear and has the potential to be a possible diagnostic tool. The above research was conducted in order to determine whether exhaled nitric oxide, measured by NIOX VERO, would be a promising diagnostic tool for severity of PAH/PH.

The hypothesis was based on the preclinical data suggesting that there is decreased bioavailability of NO in the pulmonary vasculature. It was surprising to find that on the contrary, the more severe the disease the higher the exhaled NO levels. This can be explained by the potential switch of NO from a coupled form to an uncoupled form, which promotes oxidative stress (2). It is thought that oxidative stress has been described in the abnormal vasculature in PH. In a study conducted by Archer et al., it is found that lung tissue in patients with severe PH is under oxidant stress. It suggests that peroxynitrite, as well as, several 5-hydroxyeicosatetraenoic acid and 5-oxo-ETE, which is an enzymatic (5-lipoxygenase) product, are being formed in the lung tissue. The production of these enzymes in the lung tissue may explain that fact that rather than a diminished NO production, there is a reduced exhaled NO concentration (2).

The study demonstrated a strong correlation within WHO Group 4 (CTEPH), WHO Group 5 between 6MWD and FeNO, suggesting that FeNO may be a potential biomarker of disease severity particularly in these two groups. CTEPH is often a sequel of venous thromboembolism with fatal natural history; most often results from obstruction of the pulmonary vascular bed by non-resolving thromboembolism (64). As stated by Zaba et al. Increased pulmonary vascular resistance (PVR) eventually leads to

pulmonary hypertension which, in turn leads to right heart failure. In the non-occluded areas, a pulmonary arteriopathy vaguely different from that of pulmonary arterial hypertension (PAH) can develop and contribute to disease progression (64).

The molecular basis for these correlations is unclear, although it suggests that NO pathway may play a more central role in the pathogenesis of these subtypes of PH. In favor of this argument is the beneficial effect of the soluble GC stimulator riociguat in PH Group 4 (CTEPH) and the fact that it is the only treatment with proven efficacy in this disease.

Another interesting fact is that FeNO is low in mild disease, such as in patients with relatively good 6MWD and low NYHA FC. This may indicate that FeNO could be developed as a marker of early disease. Early detection of PH is key and it has been shown that treatment has the potential to lessen symptoms and improve one's quality of life if started at an earlier stage (12). Early detection of these properties including exhaled nitric oxide for both PH/PAH could lead to a better prognosis of the disease and potential treatment for it.

WHO defines a classification of pulmonary hypertension based on the cause of the disease. Their intentions are to maintain, implement and produce international health standards (18). When this classification was first created there were only two groups and now there are a total of five groups, as seen in Table 1. The classification is continually adjusted, with the increasing information gathered for each PH disease. The most recent modifications were made in Nice, France in 2013 and there will be more modifications to occur in 2018.

The New York Health Association Functional Classification was first adapted by WHO in 1998 in order to help with the evaluation of a patient's PH (34). Functional Classification has not only helped with the evaluation of a patient's status of disease but has also helped with enrollment criteria, endpoints for clinical trials and with clinical evaluation. The NYHA FC was first created as research criteria for patients with cardiac disease and has developed into a very important and widely used criterion for majority of research studies that involve ischemic and other left heart diseases. There are four functional classifications; the four classifications allow assessment of how a patient is affected by their disease. The higher the classification, Class 4 being the highest, the more severe the patient's disease is and the greater urgency the patient needs to be diagnosed in order to start the proper treatment (35).

This study compared FeNO to 6MWD because 6MWD has been shown to be a valuable surrogate of the disease in prior studies. 6MWD correlated with hemodynamics as shown by Mclaughlin and Miyamoto in their respective studies (45,49). In addition, many studies used 6MWD as a means to determine a patient's functional capacity due to its simplicity and reliability. Hence, it has been determined to be a good end point. 6MWD is sub-maximal exercise test, which assesses the patient's aerobic capacity and endurance. The distance covered over the 6-minute period evaluates the patient's performance capacity and documents how the patient's performance improves or declines.

Interestingly, this research has found significant differences between PH Groups in regards with the 6MWD, with WHO Group 3 patients showed the lowest six-minute

walk distance. This can be explained by the co-existence of both pulmonary vascular and parenchymal (or airway) disease which is associated usually with significant hypoxemia and a more significant decline in lung performance during exercise (15). Patients with PH secondary to chronic obstructive pulmonary (COPD) are classified within WHO Group 3 and have been shown to have significantly lower 6MWDs as shown by Carratu (12). Impaired gas exchange is the most commonly encountered functional abnormality in COPD patients. Initially, oxygen desaturation occurs during exercise but with progression of disease, it occurs eventually at rest, thus showing that 6MWD is a good indicator of disease severity especially in patients with COPD (12). A patient with desaturation may feel short of breath, light-headed, dizzy, and have an elevated blood pressure and a very high heart rate. All these side effects are means to stop the test and make the patient feel as if they can no longer continue the test. For this reason, WHO Group 3 may have the lowest 6MWD.

Not surprisingly, the above research has identified a significant correlation between NYHA FC and 6MWD, although there was only one patient in NYHA FC IV. Both NYHA FC and 6MWD are surrogates for PH severity; therefore a direct correlation is in accordance with the expected disease process.

Several medications affect the NO pathway and it was interesting to evaluate the impact of PAH specific medications on FeNO levels. Although treatment-naive PAH patients tended to have slightly lower FeNO levels, there were no significant differences between treated and treatment naive patients. Therefore, our study could not attribute the any effects of PAH therapies as a whole to FeNO levels.

The study conducted above did not find any differences between the WHO Groups, suggesting that the level of NO pathway impairment is similar between different etiologies, or that the abnormalities in the pulmonary vasculature are being diluted by the NO release of other tissues in the lung, such as the bronchial mucosa, or the interstitium and FeNO cannot detect with great accuracy pulmonary vascular changes in the NO pathway.

It has been acknowledged that this study has several limitations. First, each patient that presented to the clinic had to perform a FeNO then a 6-minute walk. If the patients didn't feel like they could perform the test then the patient was not included within the study. Essentially, this eliminated any patient that had the greatest severity of the disease. Therefore, the patients with the most severe PH/PAH may have not participated within the study. Second, patient's effort, practitioner error and instrument error could all have contributed to errors that may have occurred within the study. To avoid these errors, the pulmonary team underwent special training before the study and the machine was calibrated before each use. Lastly, although this study is reporting the largest number of tests to date in PH, some groups had few patients while others had over half the patient population size, making the statistical analysis prone to errors.

In conclusion, this study reports for the first time FeNO levels in all PH Groups in a relatively large cohort of patients. It is found that FeNO levels are similar in various types of PH; that they are low in less severe disease; and that their level inversely correlates with disease severity. Lastly, in CTEPH and PH Group 5 patients FeNO may

be a potential biomarker of pulmonary vascular disease that warrants further investigation.

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